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Use of microalgae and bacteria to enhance protection of gnotobiotic *Artemia* against different pathogens

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Abstract

The present study investigates the use of microalgae, probiotic and dead bacteria in gnotobiotic *Artemia* to overcome the virulence of two pathogenic bacterial strains: *Vibrio campbellii* and *Vibrio proteolyticus*. For that purpose, two strains of the microalga *Dunaliella tertiolecta* (a medium- and a good-quality microalga) and two beneficial bacteria, selected from previous well-performing *Artemia* cultures, were provided to the brine shrimp cultured under gnotobiotic conditions. The daily supplementation with *D. tertiolecta* conferred full protection to *Artemia* towards both vibrios and was apparently more efficient and stable than the use of probiotics and dead bacteria. Only when *Artemia* were cultured in sub-optimal conditions with microalgae (but not when fed ad libitum), the addition of probiotic bacteria was able to partially protect the animals against both pathogens. The contribution of dead bacteria to the protection of *Artemia* against both pathogens was more pronounced in animals cultured with poor-quality feeds.

Keywords: *Artemia*; Gnotobiotic culture; *Dunaliella tertiolecta*; Dead bacteria; *Vibrio campbellii*; *Vibrio proteolyticus*

1. Introduction

Diseases are still a major constraint to sustainable aquaculture production, especially in larviculture, being responsible for considerable economic losses (Bachère, 2003). Nowadays, the use of preventive and environment-friendly approaches, such as immunostimulants and probiotics, is becoming increasingly important in aquaculture to maintain a healthy microbial environment in the larval rearing tanks. Several immunostimulants, such as dead bacteria (Keith et al., 1992; Alabi et al., 1999; Vici et al, 2000), as well as several probiotics, are claimed to induce and build up protection against a wide range of diseases in vertebrate and invertebrate cultures. Another approach commonly used in aquaculture because of its beneficial effects to the target-organisms is the so-called "green-water technique." This technique is based on the addition of microalgae in closed water systems in the most critical times, when larvae are fragile, sensitive to environmental changes and easily stressed (Papandroulakis et al., 2001). Numerous studies have shown enhanced growth, survival or health status of marine larvae cultured with microalgae (e.g., *Isochrysis galbana*, *Tetraselmis suecica*, *Phaedactylum tricornutum*, *Dunaliella salina* or *Dunaliella tertiolecta*) (e.g., Nass et al., 1992; Reitan et al., 1997; Cahu et al., 1998; Suppamattaya et al., 2005). Different hypotheses have been suggested to explain the beneficial effects of algae to the larvae, such as (i) direct supply of nutrients (Reitan et al., 1997); (ii) stimulation of the digestive abilities of larvae (Cahu et al., 1998); (iii) influence on the bacterial population of the rearing water

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and thus contributing to the establishment of an early gut microbial flora in the larvae (Bergh et al., 1994; Skjermo and Vadstein, 1999).

However, the application of such technologies must be based on thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies looking in detail at host-microbial interactions. A key experimental strategy to study these interactions is to first define the functioning of the host in the absence of bacteria and then to evaluate the effects of adding a single or defined population of microbes, or certain compounds (i.e., under axenic or gnotobiotic conditions) (Gordon and Pesti, 1971). Marques et al. (2004a,b, 2005) have previously shown that the brine shrimp *Artemia* is instrumental in the development of a gnotobiotic test system for studying the effect of food composition on survival and growth in the presence or absence of a challenge test. The test system uses a gnotobiotic *Artemia* culture in which the microbial community is totally controlled.

The brine shrimp *Artemia* is essential as part of the live food chain for the culture of fish and shellfish larvae (Sorgeloos et al., 1986). *Artemia* can uptake a wide variety of feed particles (this organism is a continuous and non-selective particle filter feeder able to consume a maximum particle size of 25–30 μm for nauplii and 50 μm for adults), such as baker's yeast and microalgae (Dobbeleir et al., 1980; Sorgeloos et al., 1986), which can be cultured axenically (Verschuere et al., 1999, 2000). Yet, the *Artemia* feeding condition has to be taken into account when culturing this animal in gnotobiotic conditions. In fact, according to Marques et al. (2005), the performance of *Artemia* fed with poor-quality feeds can be easily improved by the addition of non-pathogenic bacteria, partly because they are used as a source of nutrients, while the effects of such bacteria are less pronounced in animals fed with better quality feeds.

The present study investigates the use of microalgae, live and dead bacteria in gnotobiotic *Artemia* to overcome the virulence of two bacterial strains: *Vibrio campbellii* and *Vibrio proteolyticus*. For that purpose, two strains of the microalga *D. tertiolecta* (a medium- and a good-quality microalga), two beneficial bacteria and dead pathogenic bacteria were provided to the brine shrimp.

2. Materials and methods

2.1. Axenic cultures of microalgae and yeast

Axenic cultures of two strains of the microalga *D. tertiolecta* (strains DT CCAP 19/6B and DT CCAP 19/27, hereafter called DT-med and DT-opt) were obtained from the Culture Collection of Algae and Protozoa Department (CCAP, Dunstaffnage Marine Laboratory, Scotland). Each strain was cultured according to the procedures developed by Marques et al. (2004b), using 10% inocula, 0.22-μm filtered aeration, continuous light, a standard Walne medium (Walne, 1967) and filtered and autoclaved seawater (FASW, 0.2 μm). Algal strains were harvested by centrifugation in the exponential growth phase. All handlings were performed in a laminar-flow hood to maintain axenity.

The wild-type strain (WT) of the baker's yeast *Saccharomyces cerevisiae* (BY4741, genotype *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was provided by the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF, University of Frankfurt, Germany). The procedures used to culture this yeast were identical to the methods described by Marques et al. (2004a,b; 2005). Yeast cultures were grown in sterile Erlenmeyer flasks with a cotton cap placed on a shaker in the dark (30 °C, 150 rpm). A complete yeast extract–peptone–dextrose medium was used to culture the yeast, containing yeast extract (1%, wt/vol; Sigma), bacteriological-grade peptone (1%, wt/vol; Sigma), and D-glucose (2%, wt/vol; Sigma). The medium was prepared in FASW sterilized by autoclaving at 120 °C for 20 min. The yeast was harvested by centrifugation in the stationary growth phase. All handlings were performed in a laminar-flow hood to maintain axenity.

Yeast and microalgal cells were resuspended in FASW and their densities were determined by measuring twice the cell concentration, using a Bürker haemocytometer. Suspensions were stored at 4 °C and used to feed *Artemia* until the end of each experiment. The three feed sources were chosen according to their nutritional quality to *Artemia*: poor-quality feed (WT yeast–enabling low *Artemia* survival and low growth), medium-quality feed (microalga DT-med–enabling intermediate values of *Artemia* survivals and growth) and good-quality feed (the microalga DT-opt–enabling high *Artemia* survival and growth) (Marques et al., 2004a,b, 2005).

2.2. Bacterial strains and growth conditions

Two bacterial strains (strain LVS 2—*Bacillus* spp.; and strain LVS 3—*Aeromonas hydrophila*, both strains were isolated from *Artemia* cultures performed in the Laboratory of Microbial Ecology and Technology, Ghent University, Belgium, by Laurent Verschuere in 1999) were selected for their positive effect towards *Artemia* (Verschuere et al., 1999, 2000; Marques et al., 2005) and examined for their ability to protect nauplii cultured in different gnotobiotic environments against two different

pathogens, namely, *V. proteolyticus* strain CW8T2 (VP), isolated from artificial feed from a sea bass hatchery in Spain (Verschuere et al., 1999, 2000; Marques et al., 2005), and *V. campbellii* strain LMG21363 (VC), isolated from the lymphoid organ of a diseased juvenile *Penaeus monodon* in Negros Island, Philippines, by C. Lavilla-Pitogo in 2002 (Soto-Rodriguez et al., 2003; Gomez-Gil et al., 2004; Marques et al., 2005). Pure cultures of the four bacterial strains were obtained from the Laboratory of Microbial Ecology and Technology, Ghent University, Belgium, and from the Laboratory of Microbiology, Ghent University, Belgium. All bacterial strains were stored at −80 °C and were cultured on marine agar (MA), according to the procedures described by Marques et al. (2005). Bacteria were harvested by centrifugation, the supernatant was discarded and the pellet was resuspended in FASW. Bacterial densities were determined by spectrophotometry ($OD_{550}=1.000$ corresponds to 1.2×10^9 cells/ml; according to the McFarland standard, BioMerieux, Marcy L'Etoile, France, and confirmed in our study for all bacterial strains). Dead bacteria were offered to *Artemia* using aliquots of autoclaved concentrated bacteria (autoclaving at 120 °C for 20 min). In order to check if the bacteria were effectively killed by autoclaving, the strains were plated after being exposed to this method. For this purpose, 100 µl of the culture medium were transferred to marine agar (MA; $n=3$). Absence of bacterial growth was monitored after incubating plates for 5 days at 28 °C. Autoclaving treatment was 100% effective, since no bacterial growth was observed on the MA after 5 days of incubation. Dead and live bacterial suspensions were stored at 4 °C until the end of each experiment.

2.3. Artemia gnotobiotic culture

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture NV, Belgium). Bacteria-free cysts and nauplii were obtained via decapsulation according to the procedures described by Marques et al. (2004a,b). During decapsulation, 0.22 µm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and the tools were previously autoclaved at 120 °C for 20 min. Decapsulated cysts were washed carefully with FASW over a 50-µm pore size sterile net and transferred to a sterile 50 ml screw cap Falcon tube (TRP®, γ-irradiated) containing 30 ml of FASW. The tube was capped and placed on a rotator at 4 cycles per min, exposed to constant incandescent light (±41 μEm^{-2}) at 28 °C for 18–20 h. Twenty axenic nauplii (Instar II) were picked and transferred to sterile 50-ml containing 30 ml of FASW, together with the amount of feed scheduled for day 1. In treatments where bacteria were used, the bacterial suspension was added at a density of approximately 5×10^6 cells/ml. Each treatment consisted of four tubes (replicates). The tubes were placed on a rotator at 4 cycles/min, exposed to constant incandescent light (±41 $\mu E\ m^{-2}$) at 28 °C, being transferred to the laminar flow just once per day for feeding. The daily feeding schedule was adapted from Marques et al. (2004b) and was intended to provide ad libitum ratios, avoiding excessive feeding not to affect the water quality in the test tubes. The total Ash Free Dry Weight (AFDW) of microalgae, yeast and bacteria added to *Artemia* in experiments 1–3 was calculated following the procedures described by Marques et al. (2004a,b).

2.4. Methods used to verify axenity

Axenity of feed, decapsulated cysts and *Artemia* cultures were checked at the end of the experiments using a combination of plating on MA ($n=2$) and live counting (staining each sample with tetrazolium salt MTT(-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide)), following the procedures of Marques et al. (2004a,b, 2005). Contaminated culture tubes were not considered for further analysis and the experiment was repeated. Plating and live counting were also used to detect contaminations in treatments where live bacteria were supplied to *Artemia*. Therefore, pure cultures of the bacterial strains, as well as the samples of *Artemia* cultures, were stained with MTT and plated on marine agar. The shape and coloration of the bacterial colonies present in the *Artemia* cultures were visually compared to the characteristics of the pure bacterial cultures. Whenever different shaped or coloured colonies were found in the samples, the results of *Artemia* performance were rejected and the experiment was repeated.

2.5. Experimental design

This study comprises three experiments (see Fig. 1). In Experiment 1, selected bacteria (LVS 2, LVS 3 or a mixture of LVS 2 and LVS 3 added in equal proportions, called MIX) were tested on *Artemia* fed with the medium-quality microalga DT-med [sub-optimally fed–exp 1A, or fed ad libitum–exp 1B (AFDW of 2260.32±66.70 µg/FT AFDW, $n=2$)] and challenged with the pathogens VC or VP. Immediately after feeding *Artemia* for the first time, an LVS (or the MIX) strain was provided only once at a concentration of 5×10^6 cells/ml (corresponding to an AFDW of 45.87±3.87 µg/FT for LVS 2, 35.17±1.53 µg/FT for LVS 3, and 40.53±2.71 µg/FT for MIX; $n=2$). Forty-eight hours later (day 3), *Artemia* cultures were experimentally

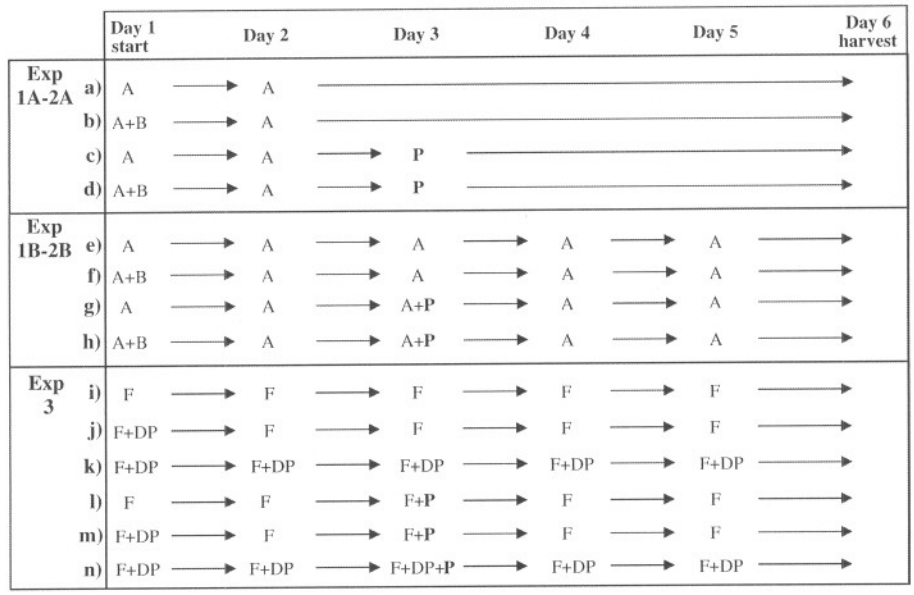

Fig. 1. Experimental design of the three experiments (Exp) performed. Legend: Exp 1A-2A—the feed was added only at day 1 and day 2; Exp 1B-2B—the feed was added daily from day 1 until day 5; (a–n) correspond to the treatments performed; A—algae (the strains DT-med or DT-opt); B—beneficial bacterium (LVS 2, LVS 3 or a mixture of equal amounts of LVS 2 and LVS 3-MIX); P—Live pathogen (*V. campbellii* or *V. proteolyticus*); F—feed provided in Exp 3: the WT yeast and the microalga DT-med; DP—Dead (autoclaved) pathogen (*V. campbellii* or *V. proteolyticus*).

infected with the same concentration of VC or VP (corresponding to an AFDW of 37.98±0.14 μg/FT for VC, and 36.02± 0.54 μg/FT for VP; $n=2$). As control treatments, *Artemia* were only supplied with the microalga without any bacteria; or supplied with the microalga and inoculated with VC or VP at day 3; or fed with the microalga and inoculated with an LVS strain at day 1 (see Fig. 1). In Experiment 2, an identical procedure to Experiment 1 was applied, changing only the feed supplied to *Artemia*: the good-quality microalga DT-opt (corresponding to an AFDW of 2710.57± 53.99 μg/FT when supplied ad libitum; $n=2$). In Experiment 3, dead VC or dead VP were offered to *Artemia* [at day 1 (corresponding to an AFDW of 33.56±0.56 μg/FT for VC, and 31.91±3.04 μg/FT for VP; $n=2$), or daily until day 6 (corresponding to an AFDW of 167.79± 2.81μg/FT for VC, and 159.55±15.22 μg/FT for VP; $n=2$)] fed with the poor-quality WT yeast (corresponding to an AFDW of 1057.95±84.76 μg/FT; $n=2$) or with the medium-quality microalga DT-med, and challenged at day 3 with the same live pathogen (similar AFDW as provided in Experiments 1 and 2). As control treatments, *Artemia* were maintained only with the feed; or maintained with the feed and infected with live VC or VP at day 3; or daily fed and supplied with dead pathogens at day 1 or daily (see Fig. 1). The experiments were performed twice to verify the reproducibility of the results. In addition, *Artemia* performance of the control treatments was compared to results previously obtained by Marques et al. (2004a,b, 2005). If significant differences were detected, data were not considered for further analysis and the experiment was repeated.

2.6. Survival and growth of Artemia

The survival percentage was determined daily for each treatment. In this way, the number of live *Artemia* was registered before feeding or adding any bacteria, by exposing each transparent Falcon tube to an incandescent light without opening the tube to avoid contamination. At the end of each experiment (day 6 after hatching), live *Artemia* were fixed with Lugol's solution to measure their individual length (IL), using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (courtesy Marnix Van Damme). As a criterion that combines both the effects of survival and IL, the total viable length (TVL) (expressed in millimetres per Falcon tube–mm/FT) was determined according to the following equation: TVL = number of survivors × mean IL.

2.7. Statistics

The percentages of larval survival were arcsine transformed, while values of IL and TVL were logarithmic or square root transformed, whenever required to satisfy normal distribution and homocedasticity requirements.

Table 1
Experiment 1—Daily survival (%), individual length (IL) (mm) and total viable length (TVL) (mm per Falcon tube—FT) of *Artemia* fed with the medium-quality microalga DT-med only until day 2 (Exp 1A) or from day 1 to day 5 (Exp 1B), inoculated with LVS 2 or LVS 3 at day 1

T	Treatments	Survival (%)					IL (mm)	TVL (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
Exp 1A—Feeding only until day 2								
a	DT-med	85 ± 10^{a}	$70\pm19^{a,b}$	65 ± 18^{a}	60 ± 16^{a}	48 ± 19^{a}	2.10 ± 0.54^{a}	$19.95\pm7.95^{a,b}$
b	DT-med+LVS2	89 ± 8^{a}	79 ± 7^{a}	53 ± 18^{a}	$46\pm10^{a,b}$	44 ± 8^{a}	2.81 ± 1.18^{a}	24.59 ± 4.22^{a}
b	DT-med+LVS3	81 ± 1^{a}	71 ± 3^{a}	$45\pm13^{a,b}$	$41\pm14^{a,b}$	39 ± 10^{a}	2.14 ± 0.60^{a}	$16.59\pm4.41^{a,b}$
c	DT-med+VC	87 ± 9^{a}	$74\pm18^{a,b}$	63 ± 17^{a}	$49\pm19^{a,b,c}$	13 ± 13^{b}	2.12 ± 0.26^{a}	5.30 ± 5.34^{c}
d	DT-med+LVS2+VC	86 ± 9^{a}	$73\pm18^{a,b}$	55 ± 17^{a}	31 ± 3^{c}	20 ± 9^{b}	2.30 ± 0.54^{a}	$9.20\pm4.20^{b,c}$
d	DT-med+LVS3+VC	81 ± 1^{a}	61 ± 3^{b}	25 ± 7^{b}	14 ± 9^{d}	13 ± 6^{b}	2.63 ± 0.55^{a}	6.58 ± 3.40^{c}
c	DT-med+VP	81 ± 9^{a}	$73\pm18^{a,b}$	50 ± 14^{a}	$40\pm14^{a,b,c}$	$21\pm15^{a,b}$	2.19 ± 0.58^{a}	$9.31\pm6.78^{b,c}$
d	DT-med+LVS2+VP	84 ± 1^{a}	$69\pm3^{a,b}$	49 ± 9^{a}	$31\pm9^{b,c,d}$	11 ± 11^{b}	2.10 ± 0.44^{a}	4.73 ± 4.66^{c}
d	DT-med+LVS3+VP	81 ± 1^{a}	61 ± 3^{b}	$41\pm13^{a,b}$	$38\pm10^{a,b,c}$	$31\pm14^{a,b}$	2.41 ± 0.82^{a}	$15.06\pm6.92^{a,b,c}$
Exp 1B—Feeding from Day 1 to 5								
e	DT-med	84 ± 9^{a}	$76\pm14^{a,b}$	$64\pm14^{a,b,c}$	$60\pm21^{a,b,c}$	$55\pm21^{a,b,c}$	1.81 ± 0.43^{a}	$19.91\pm7.68^{a,b,c}$
f	DT-med+LVS2	89 ± 8^{a}	89 ± 8^{a}	85 ± 12^{a}	85 ± 12^{a}	79 ± 9^{a}	1.85 ± 0.34^{a}	29.14 ± 3.16^{a}
f	DT-med+LVS3	78 ± 12^{a}	$76\pm13^{a,b}$	76 ± 13^{a}	76 ± 13^{a}	75 ± 14^{a}	2.05 ± 0.55^{a}	30.75 ± 5.55^{a}
g	DT-med+VC	78 ± 3^{a}	$68\pm16^{a,b}$	$40\pm15^{b,c,d}$	$36\pm17^{b,c}$	$29\pm19^{b,c}$	1.92 ± 0.34^{a}	11.04 ± 7.25^{c}
h	DT-med+LVS2+VC	88 ± 12^{a}	69 ± 10^{b}	36 ± 12^{d}	30 ± 12^{c}	23 ± 16^{c}	2.31 ± 0.65^{a}	10.40 ± 7.18^{c}
h	DT-med+LVS3+VC	83 ± 3^{a}	70 ± 7^{b}	50 ± 0^{c}	49 ± 3^{b}	$40\pm11^{b,c}$	2.21 ± 0.42^{a}	$17.68\pm4.77^{b,c}$
g	DT-med+VP	80 ± 7^{a}	$76\pm10^{a,b}$	$63\pm13^{a,b,c}$	$63\pm13^{a,b}$	$60\pm14^{a,b}$	1.89 ± 0.40^{a}	$22.68\pm5.12^{a,b,c}$
h	DT-med+LVS2+VP	84 ± 13^{a}	$73\pm17^{a,b}$	$71\pm18^{a,b}$	$66\pm21^{a,b}$	$64\pm21^{a,b}$	2.01 ± 0.61^{a}	$25.63\pm8.43^{a,b,c}$
h	DT-med+LVS3+VP	78 ± 10^{a}	$74\pm13^{a,b}$	70 ± 13^{a}	64 ± 11^{a}	63 ± 12^{a}	2.01 ± 0.45^{a}	$25.13\pm4.78^{a,b}$

VC–*V. campbellii*, VP–*V. proteolyticus*, T–Treatment, a–h correspond to the treatments performed. Data provided are the means with the standard deviation (mean±S.D.). The first column in the table is the treatment type of Fig. 1. Values in the same column showing the same superscript letters are not significantly different ($p_{Tukey}>0.05$).

Differences in survival, IL and TVL of *Artemia* cultured in different conditions were investigated with *t*-test, analysis of variances (ANOVA) and Tukey's multiple comparisons range. All statistical analyses were tested at 0.05 level of probability, using the software Statistica 5.5® (Statsoft, Inc).

3. Results

3.1. Artemia fed with the medium-quality microalga

According to Table 1 (Experiment 1), the performance of *Artemia* fed only until day 2 with the medium-quality microalga DT-med (treatment a) was not significantly different than nauplii fed daily with the same microalga (treatment e) ($n=4$; $p=0.997$). The addition of selected bacteria did not improve TVL of *Artemia* fed with this microalga (treatments b and f) in comparison to the bacteria-free control (treatments a and e). *Artemia* challenged with VC or VP and not supplied with the selected bacteria (treatments g) performed as good as unchallenged *Artemia* (treatment e), when fed daily with the microalga. Yet, *Artemia* cultured under starvation conditions with this microalga and challenged with both pathogens (treatments c) presented generally lower performance in comparison to unchallenged nauplii (treatment a), although only significantly with VC. The supplementation of selected bacteria to challenged *Artemia* fed with the microalga DT-med (treatments d and h) never improved *Artemia* performance significantly. No additional improvements in *Artemia* performance were observed when nauplii were supplied with MIX in comparison to the use of a single beneficial bacterial strain (results not shown).

3.2. Artemia fed with good-quality microalga

Artemia fed with the good-quality microalga DT-opt (Table 2–Experiment 2) presented significantly lower TVL when fed only until day 2 (treatment a) in comparison to nauplii fed daily (treatment e) ($n=4$; $p=0.002$). When fed sub-optimally with DT-opt in the presence of selected bacteria (treatments b), *Artemia* performance presented no significant improvements in comparison to the bacteria-free control (treatment a). Yet, significant improvements in TVL were found with nauplii fed daily with this microalga and supplied with LVS 2 or LVS 3 (treatments f), mostly due to higher IL. *Artemia* fed only until day 2 with DT-opt, challenged with VC or VP and not supplied with the selected bacteria (treatments c) generally revealed lower TVL in comparison to the

Table 2
Experiment 2—Daily survival (%), individual length (IL) (mm) and total viable length (TVL) (mm per Falcon tube–FT) of *Artemia* fed with the good-quality microalga DT-opt only until day 2 (Exp 2A) or from day 1 to day 5 (Exp 2B), inoculated with LVS 2 or LVS 3 at day 1

T	Treatments	Survival (%)					IL (mm)	TVL (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
Exp 2A—Feeding only until day 2								
a	DT-opt	97 ± 2^{a}	94 ± 5^{a}	84 ± 11^{a}	$66\pm15^{a,b}$	51 ± 13^{a}	2.16 ± 0.34^{b}	$22.14\pm5.40^{a,b}$
b	DT-opt+LVS2	96 ± 3^{a}	$91\pm6^{a,b}$	84 ± 8^{a}	74 ± 14^{a}	51 ± 13^{a}	2.37 ± 0.43^{b}	24.29 ± 6.23^{a}
b	DT-opt+LVS3	95 ± 4^{a}	$90\pm8^{a,b}$	81 ± 3^{a}	64 ± 5^{a}	$29\pm17^{a,b,c}$	$2.50\pm0.46^{a,b}$	$14.38\pm8.26^{a,b,c}$
c	DT-opt+VC	98 ± 2^{a}	95 ± 4^{a}	86 ± 5^{a}	39 ± 10^{c}	3 ± 5^{d}	$2.67\pm0.20^{a,b}$	1.34 ± 2.67^{d}
d	DT-opt+LVS2+VC	95 ± 2^{a}	$90\pm4^{a,b}$	79 ± 5^{a}	$43\pm10^{b,c}$	$10\pm8^{c,d}$	2.56 ± 0.27^{b}	$5.12\pm4.18^{c,d}$
d	DT-opt+LVS3+VC	95 ± 4^{a}	$90\pm8^{a,b}$	73 ± 13^{a}	$49\pm14^{a,b,c}$	$15\pm7^{b,c}$	$2.71\pm0.56^{a,b}$	8.13 ± 3.83^{c}
c	DT-opt+VP	94 ± 3^{a}	$88\pm6^{a,b}$	79 ± 14^{a}	$45\pm11^{b,c}$	3 ± 3^{d}	3.00 ± 0.17^{a}	1.50 ± 1.73^{d}
d	DT-opt+LVS2+VP	96 ± 3^{a}	$93\pm6^{a,b}$	88 ± 9^{a}	$49\pm8^{b,c}$	$13\pm9^{b,c,d}$	$2.64\pm0.33^{a,b}$	$6.60\pm4.57^{c,d}$
d	DT-opt+LVS3+VP	83 ± 4^{b}	81 ± 7^{b}	44 ± 6^{b}	41 ± 9^{c}	$29\pm9^{a,b}$	2.27 ± 0.45^{b}	$13.05\pm3.88^{b,c}$
Exp 2B—Feeding from Day 1 to 5								
e	DT-opt	93 ± 6^{a}	$93\pm6^{a,b,c}$	$91\pm5^{a,b,c}$	$91\pm5^{a,b}$	90 ± 4^{a}	2.08 ± 0.45^{c}	37.44 ± 1.70^{c}
f	DT-opt+LVS2	96 ± 5^{a}	$96\pm5^{a,b}$	$96\pm5^{a,b}$	96 ± 5^{a}	96 ± 5^{a}	$2.22\pm0.39^{b,c}$	42.74 ± 2.13^{b}
f	DT-opt+LVS3	95 ± 4^{a}	$94\pm5^{a,b,c}$	$94\pm5^{a,b,c}$	93 ± 3^{a}	91 ± 5^{a}	$2.38\pm0.41^{b,c}$	43.44 ± 2.28^{b}
g	DT-opt+VC	91 ± 9^{a}	$85\pm7^{b,c}$	81 ± 9^{c}	74 ± 11^{c}	$73\pm10^{b,c}$	$2.29\pm0.42^{b,c}$	$33.21\pm4.39^{c,d}$
h	DT-opt+LVS2+VC	93 ± 6^{a}	$91\pm5^{a,b,c}$	$84\pm8^{b,c}$	$78\pm10^{b,c}$	63 ± 9^{c}	$2.25\pm0.40^{b,c}$	28.13 ± 3.90^{d}
h	DT-opt+LVS3+VC	94 ± 3^{a}	90^{c}	$86\pm5^{b,c}$	81 ± 3^{c}	$71\pm8^{b,c}$	2.13 ± 0.39^{c}	30.03 ± 3.59^{d}
g	DT-opt+VP	98 ± 4^{a}	$95\pm7^{a,b,c}$	$95\pm7^{a,b,c}$	95 ± 7^{a}	95 ± 7^{a}	$3.07\pm0.64^{a,b}$	58.33 ± 4.34^{a}
h	DT-opt+LVS2+VP	98 ± 4^{a}	$95\pm7^{a,b,c}$	$91\pm9^{a,b,c}$	$91\pm9^{a,b,c}$	$88\pm16^{a,b}$	3.70 ± 0.69^{a}	64.75 ± 11.50^{a}
h	DT-opt+LVS3+VP	98 ± 2^{a}	$96\pm5^{a,b}$	$94\pm5^{a,b,c}$	$89\pm11^{a,b,c}$	$89\pm11^{a,b}$	3.61 ± 0.62^{a}	64.08 ± 8.00^{a}

VC–*V. campbellii*, VP–*V. proteolyticus*; T–Treatment; a–h correspond to the treatments performed. Data provided are the means with the standard deviation (mean±S.D.). The first column in the table is the treatment type of Fig. 1. Values in the same column showing the same superscript letters are not significantly different ($p_{Tukey}>0.05$).

pathogen-free control (treatment a). Yet with DT-opt fed ad libitum to *Artemia*, bacteria-free nauplii (treatment e) showed no significant differences in TVL in comparison to nauplii challenged with VC (treatment g), while nauplii challenged with VP even increased significantly TVL (treatment g), due to higher IL. Generally, the inoculation of selected bacteria to challenged nauplii fed daily with DT-opt (treatments h), could not significantly alter their TVL in comparison to treatments where only pathogens were provided (treatments g). Yet, *Artemia* fed sub-optimally with DT-opt, challenged with VC or VP and supplied with the selected bacteria (treatments h) generally revealed higher TVL in comparison to treatments where only pathogens were provided (treatments c), although only significantly with LVS 3 (treatments d), mostly due to improvements in survival). No additional improvements in *Artemia* performance were observed when nauplii were supplied with MIX in comparison to the use of a single beneficial bacterial strain (results not shown).

3.3. *Artemia fed with dead pathogens*

Performance of *Artemia* (challenged or not with live pathogens) fed with a major feed source (WT yeast or the microalga DT-med) and with dead VC or VP is presented in Table 3 (Experiment 3). Nauplii fed with both the major feed sources and with dead VC or VP at day 1 (treatments j) or daily (treatments k) presented generally significantly higher *Artemia* TVL in comparison to nauplii fed solely with the major feed source (treatments i), due both to improvements in survival and IL. However, this improvement was less pronounced with the microalga DT-med than with the WT yeast. In terms of AFDW, the highest ratio obtained between amounts of dead pathogens and amounts of the major feed source provided to *Artemia* was relatively low (0.012–0.031 when the dead pathogens were added solely at day 1, and 0.056–0.137 when added daily until day 5). *Artemia* fed with WT yeast and challenged with VP (treatment l) could survive until day 6, revealing no significant differences in TVL in comparison to the bacteria-free control (treatment i), while all nauplii fed with the same yeast and challenged with VC died before day 5 (treatment l). The addition of dead VC or VP at day 1 (or daily) to *Artemia* challenged with the same pathogens (treatments m and n) generally induced a higher TVL at the end of the experiment in comparison to challenged nauplii not supplied with dead bacteria. Yet, significantly higher survival was only registered at day 4 (in the 4

Table 3
Experiment 3—Mean daily survival (%), individual length (IL) (mm) and total viable length (TVL) (mm per Falcon tube–FT) of *Artemia* fed with wild-type yeast (WT) cultured in YEPD or with the microalga D. tertiolecta (DT-med strain)

T	Experiment 3	Survival (%)					IL (mm)	TVL (mm/FT)
		Day 2	Day 3	Day 4	Day 5	Day 6		
i	WT	57 ± 2^{c}	48 ± 5^{e}	23 ± 6^{c}	10 ± 4^{e}	8 ± 3^{d}	1.61 ± 0.16^{b}	2.42 ± 0.93^{d}
j	WT+dead VC D1	83 ± 5^{b}	$65\pm11^{c,d}$	60 ± 12^{b}	$46\pm8^{c,d}$	40 ± 0^{c}	$1.71\pm0.34^{a,b}$	13.68 ± 0.00^{c}
k	WT+dead VC	$90\pm7^{a,b}$	$90\pm7^{a,b}$	90 ± 7^{a}	90 ± 7^{a}	88 ± 6^{a}	2.19 ± 0.38^{a}	38.33 ± 2.83^{a}
l	WT+live VC D3	58 ± 5^{c}	$45\pm11^{d,e}$	1 ± 3^{d}	0^{f}	0^{f}	–	0.00^{e}
m	WT+dead VC D1+live VC D3	78 ± 4^{b}	$55\pm7^{d,e}$	30 ± 7^{c}	0^{f}	0^{f}	–	0.00^{e}
n	WT+dead VC+live VC D3	81 ± 3^{b}	81 ± 3^{b}	60 ± 8^{b}	$30\pm15^{c,d}$	$5\pm6^{d,e,f}$	1.55 ± 0.16^{b}	$1.55\pm1.79^{d,e}$
j	WT+dead VP D1	$88\pm4^{a,b}$	$76\pm9^{b,c}$	64 ± 14^{b}	$54\pm10^{b,c}$	39 ± 5^{c}	$1.75\pm0.29^{a,b}$	13.56 ± 1.68^{c}
k	WT+dead VP	$86\pm6^{a,b}$	$84\pm9^{a,b,c}$	$70\pm8^{a,b}$	61 ± 7^{b}	56 ± 7^{b}	$1.99\pm0.40^{a,b}$	22.39 ± 2.80^{b}
l	WT+live VP D3	58 ± 2^{c}	46 ± 5^{d}	21 ± 8^{c}	5 ± 4^{e}	1 ± 3^{ef}	1.71^{b}	$0.43\pm0.86^{d,e}$
m	WT+dead VP D1+live VP D3	$89\pm4^{a,b}$	$79\pm9^{b,c}$	70 ± 8^{b}	34 ± 5^{d}	$5\pm4^{d,e}$	1.59 ± 0.32^{b}	1.59 ± 1.30^{d}
n	WT+dead VP+live VP D3	96 ± 1^{a}	93 ± 3^{a}	88 ± 3^{a}	68 ± 12^{b}	34 ± 11^{c}	$1.87\pm0.34^{a,b}$	12.62 ± 4.15^{c}
i	DT-med	92 ± 5^{a}	$84\pm10^{a,b}$	$84\pm10^{a,b}$	$83\pm10^{a,b}$	$75\pm15^{b,c}$	2.21 ± 0.40^{a}	$33.15\pm6.51^{b,c}$
j	DT-med+dead VC D1	93 ± 3^{a}	$85\pm6^{a,b}$	$84\pm5^{a,b}$	$84\pm5^{a,b}$	$75\pm11^{b,c}$	2.16 ± 0.38^{a}	$32.40\pm4.67^{b,c}$
k	DT-med+dead VC	98 ± 3^{a}	95 ± 4^{a}	93 ± 3^{a}	90 ± 4^{a}	$88\pm3^{a,b}$	2.70 ± 0.57^{a}	38.36 ± 2.24^{b}
l	DT-med+live VC D3	90 ± 4^{a}	80 ± 8^{b}	80 ± 8^{b}	$79\pm10^{a,b}$	64 ± 15^{c}	2.13 ± 0.39^{a}	27.16 ± 6.36^{c}
m	DT-med+dead VC D1+live VC D3	97 ± 4^{a}	$94\pm8^{a,b}$	$94\pm8^{a,b}$	$88\pm10^{a,b}$	$74\pm19^{a,b,c}$	2.24 ± 0.40^{a}	$33.04\pm8.65^{b,c}$
n	DT-med+dead VC+live VC D3	89 ± 4^{a}	78 ± 9^{b}	76 ± 10^{b}	71 ± 9^{b}	66 ± 10^{c}	2.44 ± 0.40^{a}	$32.33\pm5.03^{b,c}$
j	DT-med+dead VP D1	89 ± 7^{a}	$78\pm14^{a,b}$	$76\pm15^{a,b}$	$75\pm17^{a,b}$	$71\pm21^{a,b,c}$	2.23 ± 0.45^{a}	$31.78\pm9.17^{b,c}$
k	DT-med+dead VP	96 ± 3^{a}	95 ± 4^{a}	95 ± 4^{a}	94 ± 5^{a}	93 ± 3^{a}	2.82 ± 0.61^{a}	49.35 ± 1.63^{a}
l	DT-med+live VP D3	94 ± 3^{a}	$89\pm6^{a,b}$	$89\pm6^{a,b}$	$86\pm11^{a,b}$	$80\pm8^{b,c}$	2.27 ± 0.36^{a}	$36.32\pm3.71^{b,c}$
m	DT-med+dead VP D1+live VP D3	89 ± 6^{a}	$79\pm13^{a,b}$	$78\pm14^{a,b}$	$75\pm17^{a,b}$	$74\pm19^{a,b,c}$	2.36 ± 0.51^{a}	$34.81\pm8.91^{b,c}$
n	DT-med+dead VP+live VP D3	91 ± 2^{a}	81 ± 5^{b}	80 ± 7^{b}	78 ± 5^{b}	70 ± 4^{c}	2.70 ± 0.57^{a}	37.80 ± 2.20^{b}

Nauplii were inoculated with dead *V. campbellii* (VC) or *V. proteolyticus* (VP) added only at day 1 (D1) or daily and challenged with the same live pathogen added only at day 3 (D3). T–Treatment, i–n correspond to the treatments performed. The first column in the table is the treatment type of Fig. 1. Means were put together with the standard deviation (mean±S.D.). Values in the same column showing the same superscript letter are not significantly different ($p_{Tukey}>0.05$).

treatments where dead pathogens were added) and day 5 (3 out of 4 treatments). *Artemia* fed with the microalga DT-med and challenged with both pathogens (treatments l) suffered no significant reduction in TVL in comparison to the pathogen-free control (treatment i). The addition of dead bacteria (at day 1 or daily) to *Artemia* fed with the microalga and challenged with both pathogens (treatments m and n) did not enhance significantly the animal performance in comparison to treatments where only live pathogens were provided (treatments l).

4. Discussion

Artemia fed with the microalga DT-opt performed always better than animals fed with the microalga DT-med (Tables 1 and 2; treatments e), indicating that both microalgal strains present different nutritional values to *Artemia*. Similar results were obtained by Marques et al. (2004b, 2005). Generally, the daily addition of medium or good-quality microalga to *Artemia*, without providing any selected bacteria, completely prevented detrimental effects from the opportunistic pathogen VP, while the virulence of VC was only avoided when *Artemia* were daily fed with the good-quality microalga DT-opt (Tables 1 and 2; treatments g; Table 4). Therefore, the microalga *D. tertiolecta* (especially the strain DT-opt) fed daily to *Artemia* could act as a biological control agent of infections, independently from the pathogenic bacteria used. Several hypotheses can explain the protection provided by this microalga, such as (i) extra (or better quality) nutrients present in the microalga that improved the general animal condition (Reitan et al., 1997; Suppamattaya et al., 2005); (ii) stimulation of the digestive physiology of *Artemia*, as described in the seabass *Dicentrarchus labrax* fed with microalgae (Cahu et al., 1998); (iii) production of antibacterial substances or growth promoting compounds by the microalga, as described for other microalgae, like *Tetraselmis* sp. (Kellam and Walker, 1989; Austin and Day, 1990; Austin et al., 1992; Bergh et al., 1994; Salvesen et al., 2000); (iv) some components present in *D. tertiolecta*, such as β-carotene (Abalde and Fábregas,

Table 4
Summary table of beneficial (significantly higher performance: +) and non-beneficial (no significant differences: = and significantly lower performance: ×) effects of adding live (LVS 2, LVS 3 or MIX) or dead bacteria (dead *V. campbellii*–VC, or *V. proteolyticus*–VP) on *Artemia* performance when fed with the microalga *D. tertiolecta* (DT-med and DT-opt) or with the wild-type baker's yeast (WT) and challenged with the live pathogens VC or VP, in comparison to challenged nauplii supplied solely with the feed

	Vibrio campbellii				*Vibrio proteolyticus*			
	Survival D4 (%)	Survival D6 (%)	IL (mm)	TVL (mm/FT)	Survival D4 (%)	Survival D6 (%)	IL (mm)	TVL (mm/FT)
Experiments 1 and 2–OD1-2								
DT-med								
LVS 2	=	=	=	=	=	=	=	=
LVS 3	×	=	=	=	=	=	=	=
DT-opt								
LVS 2	=	=	=	=	=	=	=	=
LVS 3	=	=	=	⊕	⊗	⊕	⊗	⊕
Experiments 1 and 2–D1-5								
DT-med								
LVS 2	=	=	=	=	=	=	=	=
LVS 3	=	=	=	=	=	=	=	=
DT-opt								
LVS 2	=	=	=	=	=	=	=	=
LVS 3	=	=	=	=	=	=	=	=
Experiment 3								
WT yeast								
Dead pathogen D1	⊕	=	nd	=	+	=	=	=
Dead pathogen	⊕	=	nd	=	+	+	=	+
DT-med								
Dead pathogen D1	=	=	=	=	=	=	=	=
Dead pathogen	=	=	=	=	=	=	=	=

The circles above the (+) and (×) mean that the feed was not protecting nauplii against the specific pathogen; nd means not determined due to absence of survivors; OD1-2 means that the feed was only supplied at day 1 and 2; D1-5 means that the feed was supplied daily from day 1 to day 5; D1, D4 and D6 means, respectively, days 1, 4 and 6.

1991), can boost the *Artemia* non-specific immune response against pathogenic bacteria conferring in this way protection to nauplii. β-Carotene (Bendich, 1989; Lall, 2000) is usually required for optimal functioning of the immune system in terrestrial and aquatic animals and is known to enhance overall disease resistance, improved health and performance in aquatic organisms (e.g., shrimp and fish). Yet, previous studies performed by Supamattaya et al. (2005) revealed that the immune parameters of the shrimp *P. monodon* (e.g., total haemocyte count, phenoloxidase activity, clearance of the pathogen from the haemolymph) were not affected when the animals were fed with different concentrations of β-carotene from *D. salina*, despite the enhancement of their resistance to the white spot syndrome virus.

In general, *Artemia* cultured in sub-optimal conditions (feeding interrupted at day 2) with medium- or good-quality microalgae were less protected against both pathogens (Tables 1 and 2; treatments c) than nauplii fed ad libitum (treatments g) (Table 4). Yet, Verschuere et al. (2000) obtained reverse results when *Artemia* were challenged with the same VP strain. These authors demonstrated a higher virulence/mortality of *Artemia* nauplii under feeding conditions, in comparison to starvation conditions. However, the feed used in the experiments performed by Verschuere et al. (2000) was an inert and irradiated feed possibly presenting lower nutritional quality than live microalgae. Our result seems to corroborate the hypothesis that a good animal condition is essential to avoid pathogenic effects of bacteria. Improvements in the condition of aquatic organisms can be achieved by balancing the diets with regard to nutritional factors (Raa, 2000). This phenomenon is identified as nutritional immunology, since some nutritional factors are so intimately linked with biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration or quality of the feeds (Raa, 2000). However, more studies are still required to understand the precise cause for such difference between the two feeding regimes, e.g., the direct analysis of the effect of microalgae on bacteria composition, analysis of *Artemia* gene expression when fed with microalgae.

The addition of probiotic bacteria was only beneficial to *Artemia* when fed in sub-optimal conditions with the microalga DT-opt (Tables 2 and 4). In such conditions, LVS 3 generally suppressed the detrimental effects from the opportunistic VP and from the virulent VC. In a different experimental set-up, similar results were obtained by Verschuere et al. (2000) in *Artemia* fed sub-optimally with an irradiated inert feed, inoculated with LVS 3 and challenged with VP. Several hypotheses can be formulated to explain such protection by LVS 3: e.g., the simple addition of extra amounts of nutrients (Intriago and Jones, 1993; Marques et al., 2005); the presence of active bacterial enzymes allowing additional digestive abilities in the intestine of *Artemia* (Intriago and Jones, 1993; Verschuere et al., 1999); the conversion of nutrients normally unavailable to *Artemia* into bacterial biomass with an appropriate particle size more available as feed source (nutrient recycling), improving in this way the water quality (Verschuere et al., 2000); the removal of toxic substances that can affect the growth and survival of *Artemia* (Verschuere et al., 1999); or the delivery of immunostimulants triggering the innate immune system, thus improving disease resistance. It was interesting to note that the addition of a mixture of probiotic bacteria (MIX) did not provide any extra protection against both pathogens than the use of a single bacterial strain.

The addition of dead (autoclaved) bacteria to non-challenged *Artemia* fed with the poor-quality WT yeast (Table 3, treatments j and k) significantly improved their performance in comparison to the bacteria-free controls, independently of the amount of dead bacteria provided. Yet, such improvement did not occur in animals fed with the medium-quality microalga DT-med. According to Marques et al. (2005), autoclaved bacteria can be an important source of nutrients to improve the performance of *Artemia*, especially when fed with poor-quality feeds.

Regarding the use of dead (autoclaved) bacteria in the challenge tests, we were able to demonstrate their beneficial effect to the protection of *Artemia* against pathogenic bacteria, although their efficiency seems to depend on the amount of dead bacteria provided and on the quality of the feed used (Tables 3 and 4). In fact, *Artemia* fed with the poor-quality WT yeast and supplied daily with dead VC or VP (treatments n) were able to resist to pathogenic bacteria. In contrast, challenged *Artemia* fed with a medium-quality microalga DT-med were not able to improve their performance when supplied with either dead pathogens, even when provided daily, likely because the animals were completely protected by the feed against both pathogens. The protection provided by dead bacteria to *Artemia* could be a consequence of an improvement in the health condition of the animals or due to the stimulation of their innate immune response. The use of dead pathogenic bacteria is presumed to stimulate the innate immune system of invertebrates, such as crustaceans, as they appear to mimic pathogens in a similar fashion to vaccines in vertebrates, avoiding in this way their detrimental effects (Smith et al., 2003). In previous studies, challenged *Penaeus monodon*, *P. japonicus* and *P. indicus*, vaccinated by injection or immersion with formalin-killed *Vibrio* sp., presented significantly lower mortalities in comparison to non-treated challenged animals (Itami et al., 1989, 1991; Alabi et al., 1999). Few studies even described the acquisition of longer lasting immune states following exposure of prawns to killed *Vibrio* sp. due to humoral factors, cellular factors and antibacterial activity (Itami et al., 1989; Alabi et al., 1999, 2000). In the present study, the use of dead bacteria (especially when added once) was not as efficient as the feeding condition to control diseases in gnotobiotic *Artemia*. The method used to obtain dead bacteria (autoclaving) could have contributed to this result, as this method has a strong potential to destroy several compounds, like vitamins, proteins and fatty acids, and to induce damages to cell membrane lipids (Marques et al., 2004a). Therefore, future studies should include less potentially destructive methods to inactivate bacteria.

5. Conclusions

The daily addition of the microalga *D. tertiolecta* (especially the strain DT-opt) was able to protect *Artemia* against opportunistic and virulent bacteria. The quality and quantity of microalga provided to *Artemia* played an important role in such protection, likely by influencing the animal condition. Under the experimental conditions described, the use of probiotic bacteria was only able to protect *Artemia* against pathogenic bacteria and improve their performance when the animals were cultured in sub-optimal conditions (but not when the animals were cultured in better conditions). The capacity of dead (autoclaved) pathogens to avoid the detrimental effects of the same live bacteria to *Artemia* seemed to depend on the amount of bacteria and on the quality of the feed used. Probiotics and dead pathogens were less effective than the use of the microalga *D. tertiolecta* as feed. Therefore, it will be interesting to clarify in which way *D. tertiolecta* protects *Artemia* against pathogenic bacteria, such as through transcriptome analysis. It is hoped that the present *Artemia* gnotobiotic test system will develop further into a test for the better understanding of the effect of this microalga, contributing to the

development of alternative strategies to fight diseases in aquaculture.

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